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Exogenous progesterone enhances ova and embryo quality following superstimulation of the first follicular wave in Nelore (*Bos indicus*) donors

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Abstract

Three experiments were conducted to evaluate the effects of exogenous progesterone on superovulatory response and ova/embryo quality in *Bos indicus* donors superstimulated during the first follicular wave (FFW). We hypothesized that exogenous progesterone during gonadotropin treatments would improve ova and embryo quality. In Experiment 1, 18 Nelore cows were randomly allocated to three groups: (1) FFW; (2) FFW plus a progesterone-releasing device (FFW+P4); and (3) control (E2+P4). Cows in the FFW groups were superstimulated beginning at synchronized ovulation, whereas cows in the control group were superstimulated after synchronization of follicular wave emergence with estradiol plus progesterone (E2+P4). There were no differences in mean (± SD) numbers of transferable embryos between FFW+P4 (8.0 ± 4.5) and control (6.7 ± 4.8) groups, but both were higher (P = 0.006) than the FFW group (0.2 ± 0.4). In Experiment 2, FFW and FFW+P4 were compared in 20 Nelore donors; exogenous progesterone increased the number of transferable embryos (3.9 ± 3.4 vs. 1.3 ± 4.1, P = 0.003). In Experiment 3, FFW and FFW+P4 were compared in 10 Nelore donors except that cows were slaughtered 12 h after pLH (Lutropin-V®, Bioniche Animal Health, Belleville, ON, Canada) treatment. More mature cumulus oocyte complex (COC) (expanded cumulus cell layers) were collected in the FFW+P4 group than in the FFW group (21.8 ± 13.1 vs. 10.8±14.7; P = 0.003). In summary, superovulatory response was satisfactory when FSH (Folltropin-V®, Bioniche Animal Health) treatment was initiated at emergence of the first follicular wave in Nelore (*Bos indicus)* donors, and the hypothesis that administration of exogenous progesterone during the treatment will improve oocyte and embryo quality was supported.

Keywords: Cattle; Nelore; First follicular wave; Progesterone; Embryos

1. Introduction

Traditionally, superstimulation of cattle is started during midcycle (i.e., 8 to 13 d after estrus) [1], because of the notion that luteolysis could be consistently and reliably induced only after full corpus luteum (CL) maturation [2], and because a greater superovulatory response had been reported when treatments were initiated during midcycle, rather than early in the cycle, i.e., 3 to 6 d after estrus [3]. However, none of these early studies accounted for follicular wave status when treatment was initiated.

Several approaches have been developed to manipulate the follicular wave, so that superstimulatory treatments could be initiated at follicular wave emergence.

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An approach that is currently applied commercially is synchronization of follicular wave emergence with estradiol (E2) given concurrent with insertion of a device containing progestagen/progesterone (P4) [4]. This approach reliably synchronizes emergence of a new follicular wave 3 to 5 d later [5,6]. Gonadotropin treatments initiated 4 d after treatment with E2+P4 resulted in superovulatory responses and embryo production comparable to treatments initiated at midcycle (i.e., days 8 to 12 of the estrous cycle) [7,8].

Initiation of gonadotropin treatments at the expected time of the wave-eliciting FSH (Folltropin-V®, Bioniche Animal Health, Belleville, ON, Canada) surge, near the time of ovulation and at the beginning of first follicular wave, is another option for superstimulation [9]. Follicles originating from the first follicular wave (FFW) had the same capacity to respond to exogenous gonadotropins as those from the second follicular wave [10]. However, the hormonal milieu in which follicles grow differs greatly between the first and second follicular waves, e.g., the follicles in the FFW grow under lower systemic concentrations of P4, due to the presence of the growing CL. Furthermore, superovulatory responses have been reported to be reduced when gonadotropin treatments were initiated when peripheral P4 concentrations were low [11,12].

The present study was designed to confirm that a satisfactory superovulatory response could be achieved when gonadotropin treatments were initiated at emergence of the first follicular wave in Nelore donors, and to test the hypothesis that the administration of exogenous progesterone during treatments would improve ova and embryo quality.

2. Materials and methods

2.1. Cows

Three experiments were conducted on a farm located in the state of Mato Grosso do Sul, Brazil. In all experiments, cycling, nonlactating, mature Nelore donor cows with a body condition score between 3 and 3.5 (1 to 5 scale) maintained on pasture, were used.

2.2. Superstimulation treatment protocols

2.2.1. Experiment 1

Nelore donor cows (N = 18) were randomly allocated to three treatment groups. Cows in FFW and FFW+P4 groups were superstimulated beginning at emergence of the first follicular wave (FFW), whereas cows in the control group were superstimulated 4 d after insertion of an intravaginal device containing 1.9 g of P4 (CIDR®, Pfizer Animal Health, São Paulo, SP, Brazil) and an im injection of 2.0 mg estradiol benzoate (EB; Index Farmaceutica, Sao Paulo Brazil), as previously reviewed [13]. Prior to initiating gonadotropin treatments at emergence of the first follicular wave, ovulation was synchronized by the insertion of an intravaginal device (CIDR) and 2.0 mg EB given im on Day 0 (i.e., day of the beginning of the experiment); $PGF_{2\alpha}$ (150 μg D-cloprostenol, Preloban®, Intervet, Boxmeer, The Netherlands) was given on Day 5, CIDR were removed on the afternoon of Day 9, and 12.5 mg of pLH (Lutropin-V®, Bioniche Animal Health, Belleville, ON, Canada) was given the morning of Day 10 (12 h after CIDR removal). Cows in the control (EB+P4) group were treated with a CIDR plus EB (given im) on Day 7. Superstimulation treatments were initiated in all three groups on Day 11. All donors were superstimulated with a total dose of 133 mg FSH (Folltropin-V®, Bioniche Animal Health), divided into equal (13.3 mg diluted in 1 mL of diluent), twice daily im injections over 5 d (Days 11 to 15). Cows in the FFW+P4 group also received a new CIDR concurrent with the first FSH treatment. On the last day of FSH treatment (Day 15), all cows received $PGF_{2\alpha}$ at the time of each FSH injection, and in the FFW+P4 and control groups, CIDR were removed in the PM. All cows received 25 mg of pLH (Lutropin-V®) 24 h after the last FSH injection (Day 16 PM) and were inseminated 12 and 24 h later (Day 17). On Day 24 (7 d after AI), one veterinarian performed nonsurgical (transcervival) uterine flushings for ova/embryo collection and evaluation. Treatment protocols are shown (Fig. 1).

2.2.2. Experiment 2

Nelore donor cows (N = 20) were randomly allocated to one of two treatment groups (FFW and FFW+P4). With the exception of the control group (which was excluded), treatments (including ova/embryo collection and evaluation) were done as described in Experiment 1.

2.2.3. Experiment 3

Ten Nelore cows (N = 10) were randomly allocated to one of two treatment groups, (FFW and FFW+P4) to be superstimulated as described in Experiment 2. However, cows were slaughtered 12 h after administration of 25 mg of pLH, and ovaries were collected for follicular aspiration and cumulus oocyte complex (COC) recovery and evaluation.

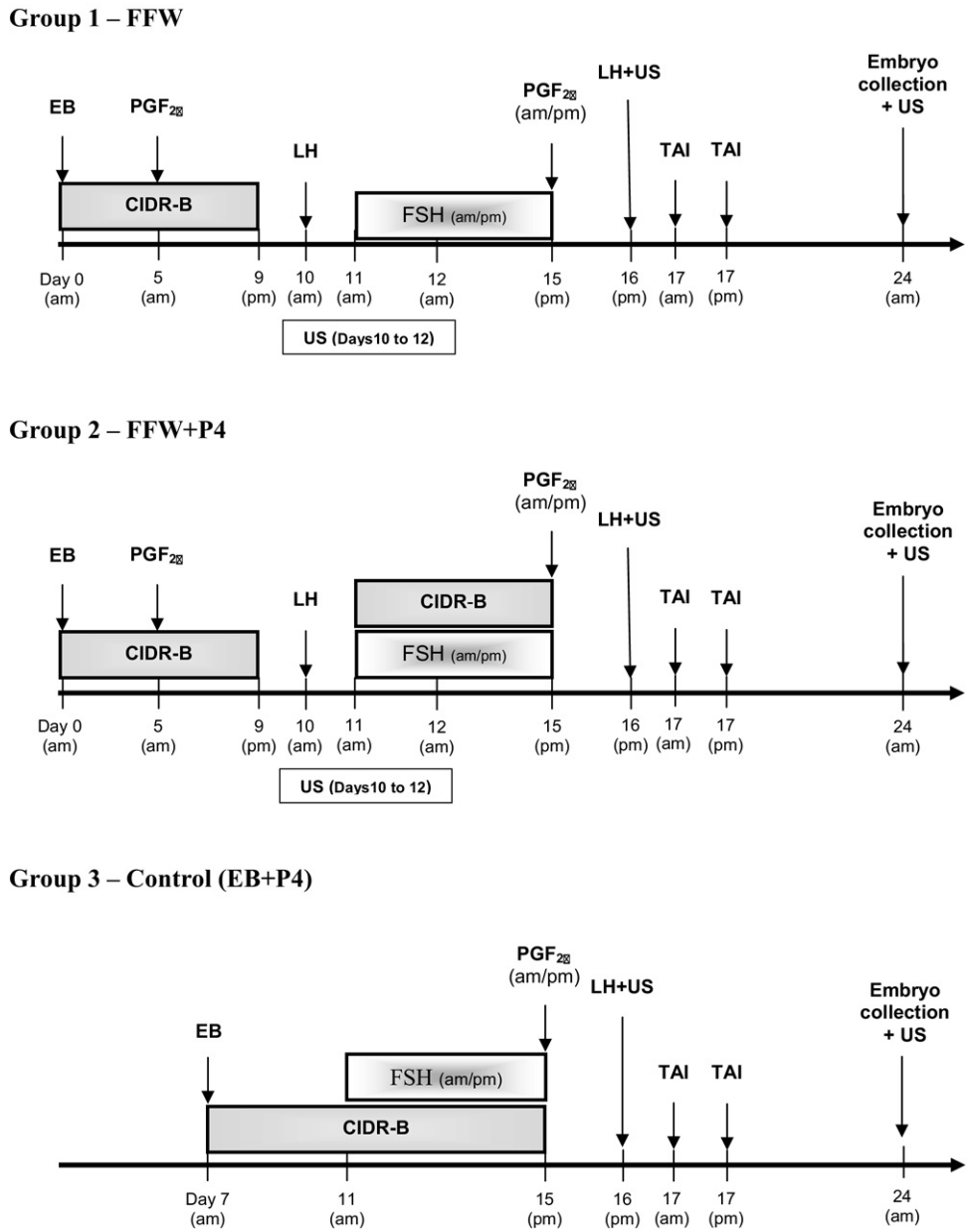

Fig. 1. Treatment protocols according to each experimental group (Experiment 1).

2.3. Ultrasound examinations

In all three experiments, transrectal ultrasonography (Aloka SSD 500, 5 MHz linear transducer, Aloka Inc., Tokyo, Japan) of both ovaries was performed in all cows from Days 10 to 12 (i.e., the day of pLH treatment to 1 d after initiation of FSH treatments) to record the presence of a preovulatory follicle and to determine the occurrence of ovulation and the emergence of the first follicular wave. In order to quantify the superovulatory response, all cows were examined ultrasonographically on Day 17 to determine the numbers of follicles >8 mm (superstimulatory response) and on Day 24 to determine the number of CL.

2.4. Embryo collection and evaluation

Ova/embryos were collected nonsurgically on Day 24 with Dulbecco's phosphate-buffered saline (PBS, Nutricell Nutrientes Celulares, Campinas, SP, Brazil)

supplemented with 1% fetal calf serum (Nutricell Nutrientes Celulares). Total ova/embryos, fertilized ova and Grades 1 (Excellent or Good), 2 (Fair), and 3 (poor) embryos were classified according to the International Embryo Transfer Society (IETS) Manual [14]. Grade 1 embryos were considered suitable to be frozen, whereas Grades 1 and 2 embryos were considered transferable.

2.5. Follicular aspiration and cumulus oocyte complex evaluation

In Experiment 3, cumulus oocyte complexes (COC) were recovered by aspirating follicles >8 mm within 1 h after slaughter, using a disposable 10 mL syringe with an 18 g needle. After recovery, COC were evaluated microscopically (magnification X 50); those with an expanded cumulus cell layer were considered mature, whereas those with compact cumulus cell layers were considered immature. Degenerate and atretic oocytes were distinguished by expanded cumulus cells layers and pyknotic, vacuolized, or partially absent oocyte cytoplasm.

2.6. Blood sampling and progesterone assay

In Experiments 2 and 3, blood samples were collected daily (Days 11 to 17) from all donors by coccygeal venipuncture into heparinized tubes (Vacutainer, Becton-Dickinson, Franklin Lakes, NJ, USA). Blood samples were centrifuged and plasma was separated and immediately stored at −20 °C for subsequent P4 analysis in the Laboratório de Dosagens Hormonais of São Paulo University. Plasma progesterone concentrations from both experiments were measured using a commercial radioimmunoassay (DPC, Diagnostic Products Corporation, Los Angeles, USA) previously validated by Garbarino et al. [15]. The intra-assay coefficient of variation was 10%. The sensitivity of the assay was 0.0076 ng/mL.

2.7. Statistical analyses

Data were analyzed by the SAS System for Windows, Version 9.2 (SAS Institute Inc., Cary, NC, USA). Data were tested for residual normality and homogeneity of variance by the Guided Data Analysis. Data transformation (Log10X or SQRT X) was employed whenever necessary.

A Poisson distribution was assumed for the categorical response variables in all three experiments. Procedure GLIMMIX was used, with the effect of donor as a random effect. The statistical model to analyze the number of follicles >8 mm at the end of FSH treatment, the number of CL at the time of ova/embryo collection, the ovulation rate, the total number of ova/embryos, the number of unfertilized ova, the number of transferable embryos, and the number of freezable embryos, included only the effect of treatment.

Plasma P4 concentrations in Experiments 2 and 3 were compared by ANOVA for repeated measures, using the MIXED procedure. The statistical model included the effects of donor, treatment, day of treatment, and treatment by day interactions.

Data are presented as mean ± SD. The level of significance to reject the hypotheses (Ho) was 5%, and a variable was considered statistically different when $P \leq 0.05$.

3. Results

3.1. Experiment 1

In Experiment 1, the number of follicles on the day of insemination (Day 17), the number of CL on the day of ova/embryo collection (Day 24), ovulation and recovery rates, and total ova/embryos did not differ among groups. However, the numbers of freezable and transferable embryos were significantly higher in the FFW+P4 and control groups than in the FFW group (Table 1).

3.2. Experiment 2

Ultrasound data in Experiment 2 confirmed that all cows ovulated prior to the initiation of the FSH treatments on Day 11. Data for other end points examined in Experiment 2 are summarized (Table 2). The numbers of follicles >8 mm on the day of insemination (Day 17) and the numbers of CL at the time of ova/embryo collection on Day 24 were higher in the FFW group than in the FFW+P4 group. Although the number of total ova/embryos did not differ, the numbers of fertilized ova, and freezable and transferable embryos were significantly higher in the FFW+P4 group than in the FFW group. There were effects of day and treatment ($P < 0.001$ for each), and day by treatment interaction ($P < 0.001$) for plasma progesterone concentrations during the superstimulatory protocol. Cows in the FFW+P4 group had higher ($P < 0.001$) plasma P4 concentrations from Day 12 to Day 15 (i.e., while the CIDR was in place) than the FFW group (Fig. 2).

3.3. Experiment 3

All cows had ovulated by 48 h after the pLH treatment. There was a significant effect of day ($P < 0.0001$), treatment ($P = 0.006$), and day by treatment interaction ($P <$

Table 1
Superovulatory response (mean ± SD) of Nelore cows treated with Folltropin-V® starting at emergence of the first follicular wave, without (FFW) or with (FFW+P4) a CIDR, or 4 d after treatment with a CIDR plus 2.0 mg estradiol benzoate im (control) (Experiment 1).

	Treatment groups			P value
	FFW	FFW+P4	Control	
Cows, N	6	6	6	—
Follicles >8 mm at AI, N	21.5 ± 16.2	18.5 ± 11.0	23.0 ± 9.2	0.26
CL at embryo collection, N	15.3 ± 10.5	12.7 ± 6.7	14.5 ± 6.1	0.47
Ovulation rate, %	74.2	74.6	63.4	0.06
Total ova/embryos	8.3 ± 7.0	11.7 ± 7.1	9.2 ± 5.6	0.19
Unfertilized ova, N	7.0 ± 6.7[a]	2.5 ± 3.9[b]	2.5 ± 3.0[b]	0.002
Transferable embryos, N	0.2 ± 0.4[a]	8.0 ± 4.5[b]	6.7 ± 4.8[b]	0.006
Freezable embryos, N	0.0 ± 0.0	5.8 ± 3.4	5.2 ± 4.3	0.89

Folltropin-V® from Bioniche Animal Health, Belleville, ON, Canada.
CL, corpus luteum.

0.0001) on plasma progesterone concentrations. Cows in the FFW+P4 group had higher plasma progesterone concentrations than the FFW group from Day 12 to Day 15 of the superstimulation protocol ($P < 0.0001$). There was no difference in the numbers of COC recovered following aspiration of all follicles >8 mm between the FFW (18.0 ± 13.6) and the FFW+P4 (23.0 ± 11.7) groups ($P = 0.12$). However, the FFW+P4 group had more mature COC (21.8 ± 13.1) than the FFW group (10.8 ± 14.7; $P = 0.003$). There was no effect of treatment ($P = 0.46$) on the numbers of degenerate or atretic oocytes (FFW, 1.2 ± 1.1; FFW+P4, 1.8 ± 2.0).

4. Discussion

In this study, a similar superovulatory response to that obtained using the combination of estradiol plus progesterone was achieved in *Bos indicus* donors when FSH treatments were performed during the first follicular wave, as previously reported for *Bos taurus* cattle [9,16]. However, embryo quality was seriously compromised in the absence of exogenous progesterone during FSH treatments. Similarly, in lactating dairy cows superstimulated during the first follicle wave, embryo quality was improved when supplemental progesterone was added to the treatment protocol [16]. However, in a previous study involving *Bos taurus* beef cattle [9], there was no apparent difference in embryo quality when treatments were initiated at the time of emergence of either the first or second follicular wave, suggesting that an exogenous source of progesterone may not have been required [10]. However, this was not specifically evaluated in this study. Therefore, the effects of supplemental progesterone should be critically

Table 2
Superovulatory response (mean ± SD) of Nelore cows treated with Folltropin-V® starting at emergence of the first follicular wave, without (FFW) or with (FFW+P4) a CIDR during the superstimulatory treatment (Experiment 2).

	Treatment groups		P value
	FFW	FFW+P4	
Cows, N	10	10	
Follicles >8 mm at AI, N	19.9 ± 6.9	14.5 ± 11.2	0.01
CL at embryo collection, N	14.6 ± 5.4	9.1 ± 6.8	0.002
Ovulation rate, %	76.9	69.2	0.06
Total ova/embryos	4.0 ± 4.8	4.7 ± 3.5	0.46
Unfertilized ova, N	2.5 ± 2.4	0.2 ± 0.6	0.003
Transferable embryos, N	1.3 ± 4.1	3.9 ± 3.4	0.003
Freezable embryos, N	1.2 ± 3.8	3.0 ± 2.9	0.02

Folltropin-V® from Bioniche Animal Health, Belleville, ON, Canada.
CL, corpus luteum.

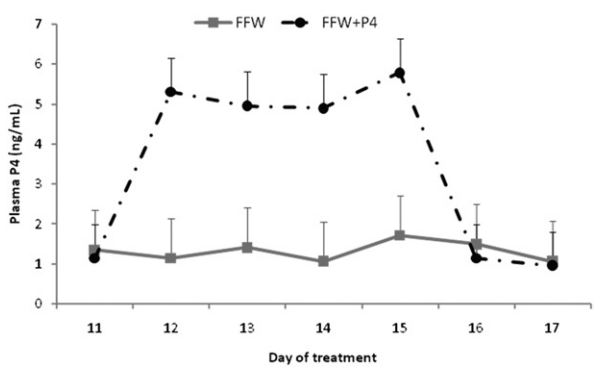

Fig. 2. Mean (± SD) plasma progesterone (P4) concentrations in Nelore cows superstimulated during the first follicular wave without (FFW) or with (FFW+P4) the insertion of a CIDR during treatment (Experiment 2). There was an effect of treatment with the CIDR ($P < 0.001$) and day of treatment ($P < 0.0001$), and an interaction between treatment and the day of superstimulation protocol ($P < 0.001$) on plasma P4 concentrations from Days 12 to 15.

examined in *Bos taurus* beef donors superstimulated at emergence of the first follicular wave.

In Experiment 1, there were no differences among the three groups in the numbers of follicles that responded to FSH treatment, numbers of CL counted on the day of ova/embryo collection, ovulation rate, embryo recovery rate, or total numbers of ova/embryos, demonstrating the capability of follicles in the first follicular wave or in an estradiol/progesterone-induced wave to respond to gonadotropin treatments. As both groups were superstimulated during the first follicular wave in Experiment 2, there was no difference in the numbers of total ova/embryos, as was expected. Despite a numerically lower superovulatory response in Experiment 2 than in Experiment 1, embryo quality was again adversely affected in cows that did not receive a progesterone-releasing device during superstimulatory treatments, confirming the results of the first experiment.

A possible cause of the reduced embryo quality in the FFW group was documented in Experiment 3; when exogenous progesterone was not provided during superstimulation of the FFW, COC did not respond to an exogenous LH injection by expansion of cumulus cells. Despite the similar numbers of COC recovered in Experiment 3, fewer mature (expanded) COC were obtained in the FFW group. The lower quality of COC in the FFW group could explain reduced embryo quality when exogenous progesterone supplementation was not supplied in Experiments 1 and 2. It has been reported that the optimal and reciprocal exchanges between the oocyte and cumulus cells are key factors to successful maturation, fertilization, and early embryo development [17]. Based on these results, cumulus cells may be considered an important parameter of oocyte competence [18,19]. Additionally, metalloproteases produced by granulosa cells after the preovulatory LH surge were responsible for degradation of proteoglycans present in gap junctions of COC [20]. Therefore, the lower incidence of cumulus cell expansion in the FFW group may have been related to an intrinsic inability of the oocyte to proceed with maturation, thereby reducing oocyte competence and subsequent embryo development. Although COC were not critically examined in this study, developmental competence was apparently compromised in FFW groups that did not receive exogenous progesterone.

It has been reported previously that superstimulation in an environment of low progesterone concentrations resulted in fewer embryos of reduced quality [11,12]. Progesterone exerts a negative feedback on the pulsatility of LH, and the latter stimulates growth and steroidogenesis of dominant follicles [21]. Low peripheral progesterone concentrations have been reported to result in aberrant LH profiles, which may interfere with oocyte maturation, ovulation, luteinization, and progesterone production, by the ensuing CL [22]. Furthermore, low progesterone concentrations could increase LH pulsatility, which may induce disturbances in nuclear maturation, reducing embryo quality and fertility [23–29].

We concluded that the lower embryo production in the FFW group in the present study was related to peripheral progesterone concentrations that were insufficient to regulate LH pulsatility. In a recent study in which cyclic dairy cows were timed-inseminated after induction of ovulation of the first wave dominant follicle, pregnancy rates were reduced and were similar to anovular cows [30]. Conversely, Denicol et al. [31] described higher pregnancy rates per AI in cows induced to ovulate the first wave dominant follicle following supplementation with exogenous progesterone during the development of that follicle compared with unsupplemented cows. Bisinotto et al. [30] also suggested that induction of ovulation of the first wave dominant follicle may compromise oocyte and embryo quality, due to the low concentrations of progesterone during development of the ovulatory follicle. However, it was noteworthy that in the present study, the adverse effects of low endogenous progesterone concentrations were minimized by providing exogenous progesterone during the superstimulatory treatment protocol.

In Experiment 2, initiation of gonadotropin treatments at emergence of the first follicular wave without supplementation with exogenous progesterone resulted in more follicles >8 mm at the time of AI, as well as more ovulations. Although not statistically significant, there were numerically more follicles and ovulations in the FFW group than in the FFW+P4 group in Experiment 1. As an alternative explanation for reduced embryo quality, the greater superstimulatory response in the FFW group may have resulted in higher estradiol blood concentrations, which in association with low peripheral progesterone concentrations due to the growing CL may have interfered with the oviductal environment, impairing fertilization and subsequent embryo development [31–34] due to a reduction on the secretory activity of the oviductal epithelial cells [17]. This also requires further study.

In a previous study in *Bos taurus* beef cattle [9], when superstimulatory treatments were initiated 24 h after ovulation (i.e., 1 d after follicle wave emergence),

superovulatory response was significantly lower than when treatments were initiated at the time of ovulation (the day of follicular wave emergence) [10]. In that study, estrus was used as a point of reference for impending ovulation and the emergence of the first follicular wave. In order to determine the time of ovulation, it was necessary to observe cows closely for signs of estrus and to do ultrasound examinations twice daily to determine the time of ovulation [9,10]. This level of management made the protocol impractical for use in large scale embryo transfer programs. In the present study, ovulation was induced with exogenous LH following synchronization of follicle growth with progesterone and estradiol benzoate. Ultrasound examinations confirmed that all donors ovulated within 48 hours after pLH treatment, providing a practical alternative for the initiation of superstimulatory treatments at the most appropriate time, without the necessity of estrus detection or frequent ultrasonographic examinations.

In summary, results of this study confirmed that superstimulation during the first follicular wave in Nelore donors results in a comparable superovulatory response relative to cows superstimulated at the time of emergence of a wave induced with a combination of estradiol and progesterone. Results also supported the hypothesis that supplementation with exogenous progesterone during superstimulatory treatments initiated at emergence of the first follicular wave improved ova and embryo quality.

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